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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 January 2003 (30.01.2003)

PCT

(10) International Publication Number
WO 03/008600 A2

(51) International Patent Classification[7]: C12P

(21) International Application Number: PCT/EP02/07349

(22) International Filing Date: 3 July 2002 (03.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

101 35 051.1	18 July 2001 (18.07.2001)	DE
60/306,867	23 July 2001 (23.07.2001)	US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

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WO 03/008600 A2

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB, or nucleotide sequences which code for these, is or are attenuated, in particular eliminated, b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid.

Process for the Preparation of L-Amino Acids using Strains of the Enterobacteriaceae Family

Field of the Invention

This invention relates to a process for the preparation of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which at least one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB is (are) attenuated.

Prior Art

L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α-

amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

Summary of the Invention

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which at least one or more of the nucleotide sequence(s) which code(s) for the genes malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB is (are) attenuated.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-

isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

The process comprises carrying out the following steps:

a) fermentation of microorganisms of the Enterobacteriaceae family in which at least one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB is (are) attenuated,

b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and

c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its

entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIIgenetika MG442
Escherichia coli VNIIgenetika M1
Escherichia coli VNIIgenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example

Serratia marcescens HNr21
Serratia marcescens TLr156
Serratia marcescens T2000.

Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of:

resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form, enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after attenuation, in particular elimination, of at least one or more of the genes chosen from the group consisting of malE,

phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB.

The nucleotide sequences of the genes of Escherichia coli belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453-1462 (1997)).

malE gene:
Description: Perimplasmic binding protein for maltose transport
Reference: Duplay et al.; Journal of Biological Chemistry 259(16): 10606-10613 (1984); Dassa and Lambert; Research in Microbiology 148(5): 389-395 (1997); Quiocho et al.; Structure 5(8): 997-1015 (1997)
Accession No.: AE000476
Alternative gene name: malB

phoA gene:
Description: Alkaline phosphatase
EC No.: 3.1.3.1
Reference: Berg; Journal of Bacteriology 146(2): 660-667 (1981)
Accession No.: AE000145

phoB gene:
Description: Regulatory protein of the pho regulon
Reference: Makino et al.; Journal of Molecular Biology 190(1): 37-44 (1986); Mc Cleary; Molecular Microbiology 20(6): 1155-1163 (1996); Torriani; Bioessays 12(8): 371-376 (1990)
Accession No.: AE000146
Alternative gene names: phoRc, phoT

phoR gene:
Description: Sensor protein of the pho regulon
EC No.: 2.7.3.-
Reference: Makino et al.; Journal of Molecular Biology 192(3): 549-556 (1986); Torriani; Bioessays 12(8): 371-376 (1990); Yamada et al.; Molecular and General Genetics 220(3): 366-372 (1990)
Accession No.: AE000146
Alternative gene names: R1pho, nmpB, phoR1

phoE gene:
Description: Pore protein E of the outer cell membrane
Reference: Overbeeke et al.; Journal of Molecular Biology 163(4): 513-532 (1983); Cowan et al.; Nature 358 (6389): 727-733 (1992)
Accession No.: AE000132
Alternative gene name: ompE

phnC gene:
Description: ATP-binding protein of the alkyl phosphonate transport system
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnD gene:
Description: Substrate-binding protein of the alkyl phosphonate transport system
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482
Alternative gene name: psiD

phnE gene:
Description: Permease protein of the alkyl phosphonate transport system

Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnF gene:
Description: Putative regulatory protein
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnG gene:
Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnJ gene:
Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnK gene:
Description: ATP-binding protein of the phosphonate transporter
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnL gene:
Description: ATP-binding protein of the phosphonate transporter
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnM gene:
Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnN gene:
Description: ATP-binding protein of the phosphonate transporter
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnO gene:
Description: Putative regulator of the carbon-phosphorus lyase complex
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

phnP gene:
Description: Membrane-bound sub-unit of the carbon-phosphorus lyase complex
Reference: Makino et al.; Journal of Bacteriology 173(8): 2665-2672 (1991)
Accession No.: AE000482

pykF gene:
Description: Fructose-stimulated pyruvate kinase I
EC No.: 2.7.1.40
Reference: Ponce et al.; Journal of Bacteriology 177(19): 5719-5722 (1995); Valentini et al.; Journal of Biological Chemistry 275(24): 18145-18152 (2000)
Accession No.: AE000262

pfkB gene:

Description: 6-Phosphofructokinase isoenzyme 2
EC No.: 2.7.1.11
Reference: Daldal; Gene 28(3): 337-342 (1984); Guixe; Archieves of Biochemistry and Biophysics 376(2): 313-319 (2000); Torres et al.; Biochemical Journal 327(3): 675-684 (1997)
Accession No.: AE000267

eda gene:
Description: 2-Keto-3-deoxygluconate 6-phosphate aldolase and 2-keto-4-hydroxyglutarate aldolase (Entner-Doudoroff aldolase)
EC No.: 4.1.2.14
4.1.3.16
Reference: Carter et al.; Gene 130(1): 155-156 (1993)
Accession No.: AE000279
Alternative gene names: hga, kdgA

talB gene:
Description: Transaldolase B
EC No.: 2.2.1.2
Reference: Sprenger et al.; Journal of Bacteriology 177(20): 5930-5936 (1995); Jia et al.; Structure 4(6): 715-724 (1996); Jia et al.; Protein Science 6(1): 119-124 (1997)
Accession No.: AE000111

rpiB gene:
Description: Ribose 5-phosphate isomerase B
EC No.: 5.3.1.6
Reference: Sorensen and Hove-Jensen; Journal of Bacteriology 178(4): 1003-1011 (1996)
Accession No.: AE000482

zwf gene:
Description: Glucose 6-phosphate 1-dehydrogenase
EC No.: 1.1.1.49

Reference: Rowley and Wolf; Journal of Bacteriology 173(3): 968-977 (1991)
Accession No.: AE000279

mopA gene:
Description: Chaperone GroEL, heat shock protein Hsp60
Reference: Chandrasekhar et al.; Journal of Biological Chemistry 261(26): 12414-12419 (1986)
Accession No.: AE000487
Alternative gene names: groE, groEL, hdh, tabB

pstA gene:
Description: Permease protein of the high-affinity phosphate transport system
Reference: Surin et al.; Journal of Bacteriology 161(1): 189-198 (1985); Amemura et al.; Journal of Molecular Biology 184(2): 241-250 (1985)
Accession No.: AE000449
Alternative gene names: R2pho, phoR2b, phoT

pstB gene:
Description: ATP-binding component of the high-affinity phosphate transport system
Reference: Surin et al.; Journal of Bacteriology 161(1): 189-198 (1985), Amemura et al.; Journal of Molecular Biology 184(2): 241-250 (1985)
Accession No.: AE000449
Alternative gene name: phoT

pstC gene:
Description: Permease protein of the high-affinity phosphate transport system
Reference: Surin et al.; Journal of Bacteriology 161(1): 189-198 (1985)
Accession No.: AE000449
Alternative gene name: phoW

pstS gene:

Description: Perimplasmic phosphate-binding protein of the high-affinity phosphate transport system

Reference: Surin et al., Journal of Bacteriology 157(3): 772-778 (1984); Magota et al., Journal of Bacteriology 157(3): 909-917 (1984)

Accession No.: AE000449

Alternative gene names: R2pho, nmpA, phoR2a, phoS

ugpA gene:

Description: Permease protein of the sn-glycerol 3-phosphate transport system

Reference: Overduin et al.; Molecular Microbiology 2(6): 767-775 (1988); Xavier et al.; Journal of Bacteriology 177(3): 699-704 (1995)

Accession No.: AE000421

Alternative gene names: psiB, psiC

ugpE gene:

Description: Permease protein of the sn-glycerol 3-phosphate transport system

Reference: Overduin et al.; Molecular Microbiology 2(6): 767-775 (1988); Xavier et al.; Journal of Bacteriology 177(3): 699-704 (1995)

Accession No.: AE000421

ugpC gene:

Description: ATP-binding component of the sn-glycerol 3-phosphate transport system

Reference: Overduin et al.; Molecular Microbiology 2(6): 767-75 (1988); Xavier et al.; Journal of Bacteriology 177(3): 699-704 (1995); Hekstra and Tommassen; Journal of Bacteriology 175(20): 6546-6552 (1993)

Accession No.: AE000421

ugpQ gene:
Description: Glycerol phosphodiester phosphodiesterase
EC No.: 3.1.4.46
Reference: Kasahara et al.; Nucleic Acids Research 17(7): 2854 (1989); Brzoska and Boos; FEMS Microbiology Reviews 5(1-2): 115-124 (1989)
Accession No.: AE000421

dnaK gene:
Description: Autoregulated heat shock protein Hsp70
Reference: Bardwell and Craig; Proceedings of the National Academy of Sciences of the United States of America 81(3): 848-52 (1984)
Accession No.: AE000112
Alternative gene names: gro, groP, groPAB, groPC, groPF, grpC, grpF, seg

dnaJ gene:
Description: Chaperone; heat shock protein
Reference: Ohki et al.; Journal of Biological Chemistry 261(4): 1778-1781 (1986)
Accession No.: AE000112
Alternative gene names: groP, grpC

clpB gene:
Description: Heat shock protein f84.1
Reference: Kitagawa et al.; Journal of Bacteriology 173(14): 4247-4253 (1991)
Accession No.: AE000345
Alternative gene name: htpM

rpoE gene:
Description: Sigma E sub-unit of RNA polymerase
Reference: Raina et al.; EMBO Journal 14(5): 1043-1055 (1995)
Accession No.: AE000343

Alternative gene name: sigE

rseA gene:
Description: Membrane protein with anti-sigmaE activity
Reference: Missiakas et al.; Molecular Microbiology 24(2): 355-371 (1997); De Las Penas et al.; Molecular Microbiology 24(2): 373-385 (1997); Collinet et al.; Journal of Biological Chemistry 275(43): 33898-33904 (2000)
Accession No.: AE000343
Alternative gene name: mclA

rseC gene:
Description: Regulatory protein of the sigma E factor
Reference: Missiakas et al.; Molecular Microbiology 24(2): 355-371 (1997); De Las Penas et al.; Molecular Microbiology 24(2): 373-385 (1997)
Accession No.: AE000343

htpG gene:
Description: Chaperone; heat shock protein c62.5
Reference: Spence and Georgopoulos; Journal of Biological Chemistry 264(8): 4398-4403 (1989); Bardwell and Craig; Proceedings of the National Academy of Sciences of the United States of America 84(15): 5177-5181 (1987)
Accession No.: AE000153

sodA gene:
Description: Superoxide dismutase
EC No. 1.15.1.1

Reference: Touati; Journal of Bacteriology 155(3): 1078-1087 (1983); Schrum and Hassan; Free Radical Biology and Medicine 17(3): 209-213 (1994); Benov and Fridovich; Archieves of Biochemistry and Biophysics 322(1): 291-294 (1995)
Accession No.: AE000465

ompF gene:
Description: Outer membrane protein F(= 1a; ia; B)
Reference: Inokuchi et al.; Nucleic Acids Research 10(21): 6957-6968 (1982)
Accession No.: AE000195
Alternative gene names: cmlB, coa, cry, tolF

ompC gene:
Description: Outer membrane protein C (= 1b)
Reference: Mizuno et al.; Journal of Biological Chemistry 258(11): 6932-6940 (1983)
Accession No.: AE000310
Alternative gene names: meoA, par

sucA gene:
Description: Decarboxylase sub-unit of 2-ketoglutarate dehydrogenase
EC No.: 1.2.4.2
Reference: Darlison et al.; European Journal of Biochemistry 141(2): 351-359 (1984); Cronan and Laporte; In: Neidhardt (ed), Escherichia coli and Salmonella, American Society for Microbiology, Washington, D.C., USA: 206-216 (1996)
Accession No.: AE000175
Alternative gene names: lys, met

sucB gene:
Description: Dihydrolipoyltranssuccinase sub-unit of 2-ketoglutarate dehydrogenase

EC No.: 2.3.1.61
Reference: Spencer et al.; European Journal of Biochemistry 141(2): 361-374 (1984); Cronan and Laporte; In: Neidhardt (ed), Escherichia coli and Salmonella, American Society for Microbiology, Washington, D.C., USA: 206-216 (1996)
Accession No.: AE000175
Alternative gene names: lys, met

sucC gene:
Description: β-Sub-unit of succinyl-CoA synthetase
EC No. 6.2.1.5
Reference: Buck et al.; Biochemistry 24(22). 6245-6252 (1985); Buck and Guest; Biochemical Journal 260(3): 737-747 (1989); Cronan and Laporte; In: Neidhardt (ed), Escherichia coli and Salmonella, American Society for Microbiology, Washington, D.C., USA: 206-216 (1996)
Accession No.: AE000176

sucD gene:
Description: α-Sub-unit of succinyl-CoA synthetase
EC No. 6.2.1.5
Reference: Buck et al.; Biochemistry 24(22), 6245-6252 (1985); Buck and Guest; Biochemical Journal 260(3): 737-747 (1989); Cronan and Laporte; In: Neidhardt (ed), Escherichia coli and Salmonella, American Society for Microbiology, Washington, D.C., USA: 206-216 (1996)
Accession No.: AE000176

gltA gene:
Description: Citrate synthase
EC No.: 4.1.3.7

Reference: Spencer and Guest, Journal of Bacteriology 151(2): 542-552 (1982)
Accession No.: AE000175
Alternative gene names: gluT, icdB

sdhB gene:
Description: Iron-sulfur protein sub-unit of succinate dehydrogenase
EC No.: 1.3.99.1
Reference: Darlison and Guest, Biochemical Journal 223(2), 507-517 (1984)
Accession No.: AE000175

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

The genes described in the text references mentioned can be used according to the invention. Alleles of the genes which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

To achieve an attenuation, for example, expression of the genes or the catalytic properties of the enzyme proteins can be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing, by genetic modification (mutation) of the signal structures of gene expression or also by the antisense-RNA technique. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start

codon and terminators. The expert can find information in this respect, inter alia, for example, in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), in Carrier and Keasling (Biotechnology Progress 15: 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3: 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as, for example, the textbook of Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that of Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences USA 95: 5511-5515 (1998)), Wente and Schachmann (Journal of Biological Chemistry 266: 20833-20839 (1991)). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair in a gene lead to "frame shift mutations", which lead to incorrect amino acids being incorporated or translation being interrupted prematurely. If a stop codon is formed in the coding region as a consequence of the mutation, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known

textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the genes, such as, for example, deletion mutations, can be incorporated into suitable strains by gene or allele replacement.

A conventional method is the method, described by Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), of gene replacement with the aid of a conditionally replicating pSC101 derivative pMAK705. Other methods described in the prior art, such as, for example, those of Martinez-Morales et al. (Journal of Bacteriology 181: 7143-7148 (1999)) or those of Boyd et al. (Journal of Bacteriology 182: 842-847 (2000)), can likewise be used.

It is also possible to transfer mutations in the particular genes or mutations which affect expression of the particular genes into various strains by conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family, in addition to attenuation of one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB, for one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide

phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism to be enhanced.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

Thus, for example, at the same time one or more of the genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene of Corynebacterium glutamicum which codes for pyruvate carboxylase (WO 99/18228),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),

- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)),
- the fba gene which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)),
- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),

- the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
- the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)),
- the mopB gene which codes for 10 Kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES,
- the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),
- the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995)),
- the cysK gene which codes for cysteine synthase A (Journal of Bacteriology 170: 3150-3157 (1988)),
- the cysB gene which codes for the regulator of the cys regulon (Journal of Biological Chemistry 262: 5999-6005 (1987)),
- the cysJ gene of the cysJIH operon which codes for the flavoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the cysI gene of the cysJIH operon which codes for the haemoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)) and

- the cysH gene of the cysJIH operon which codes for adenylyl sulfate reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989))

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to attenuation of one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- the poxB gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),

- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-261 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the cra gene,
- the rpoS gene which codes for the sigma^{38} factor (WO 01/05939) and is also known under the name of the katF gene,
- the aspA gene which codes for aspartate ammonium lyase (Nucleic Acids Research 13(6): 2063-2074 (1985)),
- the aceB gene which codes for malate synthase A (Nucleic Acids Research 16(19): 9342 (1988),
- the aceK gene which codes for isocitrate dehydrogenase kinase/phosphatase (Journal of Bacteriology 170(1): 89-97 (1988)) and
- the ugpB gene which codes for the periplasmic binding protein of the sn-glycerol 3-phosphate transport system (Molecular Microbiology 2(6): 767-775 (1988))

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to attenuation of one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE,

rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep

liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25ºC to 45ºC, and preferably 30ºC to 40ºC. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical

Chemistry 30: 1190-1206 (1958)), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

What is claimed is:

1. A process for the preparation of L-amino acids, in particular L-threonine, which comprises carrying out the following steps:

   a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB, or nucleotide sequences which code for these, is or are attenuated, in particular eliminated,

   b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and

   c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

2. A process as claimed in claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

3. A process as claimed in claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.

4. A process as claimed in claim 1, wherein the expression of the polynucleotide (s) which code(s) for one or more

of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB is attenuated, in particular eliminated.

5. A process as claimed in claim 1, wherein the regulatory and/or catalytic properties of the polypeptides (proteins) for which the polynucleotides malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB code are reduced.

6. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,

6.2 the pyc gene which codes for pyruvate carboxylase,

6.3 the pps gene which codes for phosphoenol pyruvate synthase,

6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,

6.5 the pntA and pntB genes which code for transhydrogenase,

6.6 the rhtB gene which imparts homoserine resistance,

6.7 the mqo gene which codes for malate:quinone oxidoreductase,

6.8 the rhtC gene which imparts threonine resistance,

6.9 the thrE gene which codes for the threonine export protein,

6.10 the gdhA gene which codes for glutamate dehydrogenase,

6.11 the hns gene which codes for the DNA-binding protein HLP-II,

6.12 the pgm gene which codes for phosphoglucomutase,

6.13 the fba gene which codes for fructose biphosphate aldolase,

6.14 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,

6.15 the ptsI gene which codes for enzyme I of the phosphotransferase system,

6.16 the crr gene which codes for the glucose-specific IIA component,

6.17 the ptsG gene which codes for the glucose-specific IIBC component,

6.18 the lrp gene which codes for the regulator of the leucine regulon,

6.19 the mopB gene which codes for 10 Kd chaperone,

6.20 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,

6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,

6.22 the cysK gene which codes for cysteine synthase A,

6.23 the cysB gene which codes for the regulator of the cys regulon,

6.24 the cysJ gene which codes for the flavoprotein of NADPH sulfite reductase,

6.25 the cysI gene which codes for the haemoprotein of NADPH sulfite reductase and

6.26 the cysH gene which codes for adenylyl sulfate reductase,

is or are enhanced, in particular over-expressed, are fermented.

7. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

7.1 the tdh gene which codes for threonine dehydrogenase,

7.2 the mdh gene which codes for malate dehydrogenase,

7.3 the gene product of the open reading frame (orf) yjfA,

7.4 the gene product of the open reading frame (orf) ytfP,

7.5 the pckA gene which codes for phosphoenol pyruvate carboxykinase

7.6 the poxB gene which codes for pyruvate oxidase

7.7 the aceA gene which codes for isocitrate lyase,

7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,

7.9 the fruR gene which codes for the fructose repressor,

7.10 the rpoS gene which codes for the sigma^{38} factor,

7.11 the aspA gene which codes for aspartate ammonium lyase,

7.12 the aceB gene which codes for malate synthase A

7.13 the aceK gene which codes for isocitrate dehydrogenase kinase/phosphatase and

7.14 the ugpB gene which codes for the periplasmic binding protein of the sn-glycerol 3-phosphate transport system

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 January 2003 (30.01.2003)

PCT

(10) International Publication Number
WO 03/008600 A3

(51) International Patent Classification[7]: C12P 13/08, 13/10, 13/22, 13/24, 13/12, 13/06, 13/14

(21) International Application Number: PCT/EP02/07349

(22) International Filing Date: 3 July 2002 (03.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 35 051.1 18 July 2001 (18.07.2001) DE
60/306,867 23 July 2001 (23.07.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
13 November 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/008600 A3

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of malE, phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and sdhB, or nucleotide sequences which code for these, is or are attenuated, in particular eliminated, b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/07349

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 C12P13/10 C12P13/22 C12P13/24 C12P13/12
C12P13/06 C12P13/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUNKE S ET AL: "ATP modulates subunit-subunit interactions in an ATP-binding cassette transporter (MalFGK2) determined by site-directed chemical cross-linking." THE JOURNAL OF BIOLOGICAL CHEMISTRY. US, vol. 275, no. 20, 19 May 2000 (2000-05-19), pages 15526-15534, XP002244007 ISSN: 0021-9258 the whole document ----- -/--	1-7

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2003	08. 09. 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kools, P.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/07349

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	IKEDA M ET AL: "TRANSPORT OF AROMATIC AMINO ACIDS AND ITS INFLUENCE ON OVERPRODUCTION OF THE AMINO ACIDS IN CORYNEBACTERIUM GLUTAMICUM" JOURNAL OF FERMENTATION AND BIOENGINEERING, SOCIETY OF FERMENTATION TECHNOLOGY, JP, vol. 78, no. 6, 1994, pages 420-425, XP000608032 ISSN: 0922-338X page 424, column 1, paragraph 4	1-7
A	OKAMOTO K ET AL: "HYPERPRODUCTION OF L-THREONINE BY AN ESCHERICHIA COLI MUTANT WITH IMPAIRED L-THREONINE UPTAKE" BIOSCIENCE BIOTECHNOLOGY BIOCHEMISTRY, JAPAN SOC. FOR BIOSCIENCE, BIOTECHNOLOGY AND AGROCHEM. TOKYO, JP, vol. 61, no. 11, November 1997 (1997-11), pages 1877-1882, XP001018682 ISSN: 0916-8451 abstract	1-7
A	LANDGRAF J R ET AL: "THE ROLE OF H-NS IN ONE CARBON METABOLISM" BIOCHIMIE, MASSON, PARIS, FR, vol. 76, no. 10/11, 1994, pages 1063-1070, XP008014239 ISSN: 0300-9084 page 1064, column 1, last paragraph	1-7
A	US 4 347 318 A (MIWA KIYOSHI ET AL) 31 August 1982 (1982-08-31) the whole document	1-7
A	EP 1 038 970 A (AJINOMOTO KK) 27 September 2000 (2000-09-27) page 2, paragraph 7	1-7
E	WO 03 008605 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 30 January 2003 (2003-01-30) the whole document	7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/07349

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7 partial

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7 partial

Process for the preparation of L-amino acids which comprises fermentation of microorganisms of the Enterobacteriaceae family in which at least the malE gene is attenuated, or eliminated, and one or more genes of the following group is/are attenuated or eliminated: phoA, phoB, phoR, phoE, phnC, phnD, phnE, phnF, phnG, phnJ, phnK, phnL, phnM, phnN phnO, phnP, pykF, pfkB, eda, talB, rpiB, zwf, mopA, pstA, pstB, pstC, pstS, ugpA, ugpE, ugpC, ugpQ, dnaK, dnaJ, clpB, rpoE, rseA, rseC, htpG, sodA, ompF, ompC, sucA, sucB, sucC, sucD, gltA and/or sdhB. Said process, in which further genes of the biosynthesis pathway are additionally enhanced. Said process in which microorganisms are employed in which the metabolic pathways which reduce the formation of the amino-acid are at least partly eliminated. Said process in which the regulatory and/or catalytic properties of the polypeptides encoded by said genes are reduced. Said process in which microorganisms in which in addition to the attenuated gene(s) one or more genes of the following group is/are enhanced: thrABC, pyc, pps, ppc, pntA and pntB, rhtB, mqo, rhtC, thrE, gdhA, hns, pgn, fba, ptsH, ptsI, crr, ptsG, lrp, mopB, ahpC, ahpF, cysK, cysB, cysJ, cysI, and/or cysH. Said process in which microorganisms in which in addition to the attenuated gene(s) one or more genes of the following group is/are attenuated: tdh, mdh, yjfA, ytfP, pckA, poxB, aceA, dgsA, fruR, rpoS, aspA, aceB, aceK and/or ugpB.

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2. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phoA gene and vice versa.

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3. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phoB gene and vice versa.

---

4. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phoR gene and vice versa.

---

5. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phoE gene and vice versa.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

6. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnC gene and vice versa.

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7. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnD gene and vice versa.

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8. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnE gene and vice versa.

---

9. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnF gene and vice versa.

---

10. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnG gene and vice versa.

---

11. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnJ gene and vice versa.

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12. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnK gene and vice versa.

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13. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnL gene and vice versa.

---

14. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnM gene and vice versa.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

15. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnN gene and vice versa.

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16. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnO gene and vice versa.

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17. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the phnP gene and vice versa.

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18. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the pykF gene and vice versa.

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19. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the pfkB gene and vice versa.

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20. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the eda gene and vice versa.

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21. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the talB gene and vice versa.

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22. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the rpiB gene and vice versa.

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23. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the zwf gene and vice versa.

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24. claims: 1-7 partial

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As in subject 1, in which the malE gene is replaced by the mopA gene and vice versa.

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25. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the pstA gene and vice versa.

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26. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the pstB gene and vice versa.

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27. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the pstC gene and vice versa.

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28. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the pstS gene and vice versa.

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29. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the ugpA gene and vice versa.

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30. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the ugpE gene and vice versa.

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31. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the ugpC gene and vice versa.

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32. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the ugpQ gene and vice versa.

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33. claims: 1-7 partial

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As in subject 1, in which the malE gene is replaced by the dnaK gene and vice versa.

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34. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the dnaJ gene and vice versa.

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35. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the clpB gene and vice versa.

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36. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the rpoE gene and vice versa.

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37. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the rseA gene and vice versa.

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38. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the rseC gene and vice versa.

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39. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the htpG gene and vice versa.

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40. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the sodA gene and vice versa.

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41. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the ompF gene and vice versa.

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42. claims: 1-7 partial

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As in subject 1, in which the malE gene is replaced by the ompC gene and vice versa.

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43. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the sucA gene and vice versa.

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44. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the sucB gene and vice versa.

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45. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the sucC gene and vice versa.

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46. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the sucD gene and vice versa.

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47. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the gltA gene and vice versa.

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48. claims: 1-7 partial

As in subject 1, in which the malE gene is replaced by the sdhB gene and vice versa.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/07349

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